VERSION WITH MARKINGS TO SHOW CHANGES MADE

APPENDIX A

FIG. 1A and FIG. 1B. Saturation Binding of [^{3}H]Diprenorphine Using COS-7 Cell Membranes. [^{3}H]Diprenorphine binding was determined using membranes prepared from COS-7 cells transfected with either the rat MOR-1 cDNA plasmid (○) or the parental vector (○). FIG. 1A shows data from a representative experiment are presented and are expressed as mean ± standard error . (FIG. 1B), [Inset,] Scatchard plot analysis of the binding data from MOR-1-transfected cells.

FIG. 2A and FIG. 2B. FIG. 2A and FIG. 2B [Panels A and B of FIG. 2] show displacement of [^{3}H]Diprenorphine Binding with Unlabeled Ligands as Competitors Data from a representative experiment are presented for each ligand. FIG. 2A, using opioid agonists as competitors; FIG. 2B, using opioid antagonists and somatostatins as competitors.

FIG. 5A and FIG. 5B FIG. 5A and FIG. 5B [Panels A and B of FIG. 5] show Saturation and Displacement Binding of Labeled Ligands Using Cell Membranes Transiently Expressing the Human μ Opioid Receptor. (FIG. 5A, top) [(A)] Saturation binding of [^{3}H]diprenorphine (DPN) was determined using membranes prepared from cDNA- transfected COS-7 cells. Data for all saturation binding experiments were analyzed using the linear/non-linear regression analysis program EBDA/LIGAND (Munson, 1983) to obtain estimates of K_d and B_{max} values. Data represent mean ± SEM of 3 separate experiments performed in duplicate. (FIG. 5A, bottom) [Inset:] Saturation binding plotted in Scatchard coordinates (representative curve shown). (FIG. 5B) Displacement of [^{3}H]DAMGO binding with unlabeled ligands as competitors. Displacement of [^{3}H]DAMGO binding was performed using 1.3 nM [^{3}H]DAMGO and unlabeled competitors with concentrations ranging from 0.01 nM to 5 μM. Data represent mean ± SEM of 3 separate experiments performed in duplicate.

FIG. 6A, FIG. 6B and FIG. 6C. FIG. 6A, FIG. 6B and FIG. 6C [Panels A, B, and C of FIG. 6] show Human μ Opioid Receptor Coupling to the G Protein-Activated K^+ Channel Electrophysiological analysis of oocytes injected with RNAs encoding the human μ opioid receptor and the G protein-activated K^+ channel. Membrane current traces were recorded at a holding potential of –80 mV. Oocytes were bathed in a high K^+ solution and were exposed to either (FIG. 6A) 1 μM DAMGO or (FIG. 6B) 1 μM DAMGO plus 10 μM naloxone. Inward current is downward. (FIG. 6C) The current-voltage relationship plotted as an I vs. V curve. DAMGO-induced membrane currents were recorded with voltage steps ranging from –160 mV to 0 mV. The DAMGO-induced net currents were derived by subtracting the currents recorded before DAMGO application from those during DAMGO application.

FIG. 7A, FIG. 7B and FIG. 7C. FIG. 7A, FIG. 7B and FIG. 7C [Panels A, B, and C of FIG. 7] show Desensitization of the Human μ Opioid Receptor-K^+ Channel Coupling and the Effect of PKC. Membrane currents were recorded in oocytes injected with both the human μ opioid receptor and the K^+ channel mRNAs. (FIG. 7A, top), a schematic diagram of the experimental protocol. The oocyte was voltage-clamped at –80 mV and superfused with 500 nM DAMGO in HK solution to elicit the K^+ current. After the first DAMGO stimulation, the superfusate was switched to ND96 containing 1.8 mM CaCl2 and the oocyte either received no treatment or was subjected to drug treatment or enzyme injection. The superfusate was then switched back to HK solution to record the second DAMGO-induced membrane current. (FIG. 7A, middle), the ramp voltage command used to record currents before and during DAMGO stimulation. From a holding potential of –80 mV, the membrane voltage was stepped to –160mV, and ramped to +40 mV before stepping back to the holding potential. Net currents were derived by subtracting the currents before DAMGO application from those during DAMGO application. (FIG. 7A,

bottom), the step voltage commands used to record currents before and during DAMGO application. The step command ranged from –160 mV to +40 mV, with 20 mV increments. Net currents were derived by subtracting the currents before DAMGO application from those during DAMGO application. (FIG. 7B), membrane currents elicited by DAMGO application before and after treatment with 100 nM phorbol 12-myristate 13-acetate (PMA) for 10-15 min. (FIG. 7B, top), current recorded at a holding potential of -80 mV. (B, middle and bottom), net currents from the first and second DAMGO applications are obtained from either ramped (middle) or stepped (bottom) voltage commands. (FIG. 7C), membrane currents recorded during DAMGO application before and after treatment with 100 nM 4α-phorbol for 10-15 min. (FIG. 7C, top/middle/bottom), membrane currents are recorded and displayed as in (FIG. 7B).

FIG. 8A and FIG. 8B. FIG. 8A and FIG. 8B [Panels A and B of FIG. 8] show Effect of CaM Kinase II on the Coupling Between the Human μ Opioid Receptor and K^+ Channel. Membrane currents were recorded in oocytes injected with both the human μ opioid receptor and K^+ channel mRNAs. Experimental protocol and voltage commands are as described in FIG. 7. (FIG. 8A, top/middle/bottom), membrane currents recorded during DAMGO application before and after injection of activated CaM kinase II. (FIG. 8A, top), current recorded at a holding potential of -80 mV. (FIG. 8A, middle and bottom), net currents from the first and second DAMGO applications are obtained from ramped (FIG. 8A, middle) or stepped (FIG. 8A, bottom) voltage commands. (FIG. 8B, top/middle/bottom), membrane currents recorded during DAMGO application before and after injection of boiled CaM kinase II. Membrane currents are recorded and displayed as in the top, middle and bottom panels of A.

FIG. 11A and FIG. 11B. Saturation binding of the transfected and the parental Chinese hamster ovary (CHO) cells. [^{3}H]DAMGO binding was performed using the membrane prepared

from the transfected (○) or the nontransfected parental CHO cells (○) (FIG. 11A). The data are representative of two saturation binding assays. Duplicate measurements were performed for each [^{3}H]DAMGO concentration used. The smooth lines represent the rectangular hyperbola fitting to the data. (FIG. 11B)[Inset:] the Scatchard plot analysis of the data for the transfected cells.

FIG. 18A and FIG. 18B. FIG. 18A and FIG. 18B [Panels A and B of FIG. 18] show Coupling of the μ opioid receptors to the G protein-activated K^+ channel. Electrophysiologic analysis of oocytes injected with MRNAs for the rat μ opioid receptor and the G protein-activated K^+ channel. (FIG. 18A) Membrane current traces recorded at a holding potential of – 80 mV. Oocytes were exposed to 1 μM of DAMGO (FIG. 18A, left trace) or 1 μM of DAMGO plus 10μM of naloxone (FIG. 18A, right trace) as indicated. Inward current is downward. (FIG. 18B) Membrane currents recorded with voltage steps ranging from – 160 mV to +40 mV were recorded before and 1 min after DAMGO superfusion. The DAMGO-induced net currents were derived by subtracting the currents before DAMGO application from those after, and are shown in the FIG. 18B, top panel[left panel]. The [right panel] FIG. 18B, bottom panel shows the I-V curve of the these currents.

FIG. 19A and FIG. 19B. FIG. 19A and FIG. 19B [Panels A and B of FIG. 19] show pertussis toxin (PTX) sensitivity of the μ opioid receptor-K^+ channel coupling. Oocytes injected with both the μreceptor and the K^+ channel mRNAs were incubated with 0.5 μg/ml of PTX for 24 hours before recording. (FIG. 19A) DAMGO-induced currents at holding potential of –80 mV in untreated and PTX-treated oocytes. Data are presented as mean ± S.E. with the sample size shown in parenthesis. Student t-test showed a significant difference ($p<0.01$). (FIG. 19B) Averaged I-V curves of the DAMGO-induced net currents from both groups.

FIG. 20A and FIG. 20B. FIG. 20A and FIG. 20B [Panels A and B of FIG. 20] show GTP-γ-S enhancement of the receptor-channel coupling. Fifty nanoliters of 1 mM GTP-γ-S or 10 mM Tris (pH 7.5, used as control) were injected after the membrane current induced by 1 μM of DAMGO reached plateau in oocytes injected with both the μ receptor and the K^+ channel mRNAs. (FIG. 20A) Two representative current traces recorded with a holding potential at –80 mV. Time point of injection was marked at the bottom of the traces. (FIG. 20B) Time course of the membrane current change after injection as compared to the value immediately before the injection. Data are the average of recordings from three oocytes in each group.

FIG. 21A and FIG. 21B. FIG. 21A and FIG. 21B [Panels A and B of FIG. 21] show Differential regulation of the coupline by PKA and PKC. Membrane currents were recorded from oocytes injected with both the μ receptor and the K^+ channel mRNAs. (FIG. 21A) A representative current trace recorded in a oocyte at a holding potential of –80 mV illustrating the experimental protocol. The cell was bathed in a high potassium (HK) solution, and 1 μM of DAMGO was applied by superfusion to elicit the K^+ current. After the first DAMGO stimulation, the superusate was switched to ND96 containing 6mM of $CaCl_2$, and the oocyte was either untreated (as in this example) or subjected to drug treatment or enzyme injection (see below). The superfusate was then switched back to HK solution to record the second DAMGO-induced membrane current. (FIG. 21B) Relative response of the DAMG)-induced membrane currents from different treatment groups at a membrane potential of –80mV. Data are expressed as the percentage of the peak current induced by second DAMGO stimulation over that of the first stimulation, and are presented as mean $\pm$ S.E. (n=4). Treatment is labeled on the bottom of each bar. Result of variance analysis is shown as (**) with $p<0.01$ as compared to the untreated group. Different treatments used in this experiment are as follows: 8-CPT-cAMP, incubation

with 1 mM of 8-CPT-cAMP for 10 min; PMA, incubation with 100 nM of PMA for 10 min; PKA, injection of the catalytic subunit of PKA (50 fmol/cell).

FIG. 23A and FIG. 23B. FIG. 23A and FIG. 23B [Panels A and B of FIG. 23] show RNA tissue distribution and Southern blot analysis of the putative opioid receptor. (FIG. 23A) RNAs from eight different rat tissues are labeled above each lane. About 2 μg of polyA(+) RNA was used for each tissue. The sizes of the RNA size marker are labeled on the left side; (FIG. 23B) Genomic DNA Southern blot analysis. The restriction enzymes used to cut the rat genomic DNA are labeled above the corresponding lanes. ΛDNA digested with *Hin*dIII was used as the size marker with their sizes labeled on the left side.

FIG. 24A and FIG. 24B. Expression of a voltage-activated calcium channel. A voltage-activated calcium channel is expressed in *Xenopus* oocytes by microinjection of a plasmid containing the cDNA for the calcium channel (Soong *et al.* 1993). Two to three days after injection, oocytes are voltage-clamped and the calcium channel expression is measured using a step protocol. The calcium channel function is determined using a solution of 40mM barium chloride and shown as barium current through the calcium channel. FIG. 24A [Top panel]: command voltage steps from a holding potential of –100 mV to either –10 mV or +10mV. FIG. 24B [Bottom panel]: transient currents evoked by the voltage steps.

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APPENDIX: B

44. (Twice Amended) A process of screening a candidate substance for its ability to interact with a mu opioid receptor comprising:

a) providing a recombinant mu opioid receptor polypeptide comprising the contiguous amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:8, or SEQ ID NO:17;

b) obtaining a candidate substance; and

c) testing the ability of said candidate substance to interact with said opioid receptor.

72. (Amended) The process of claim 65, wherein detecting the ability of the candidate substance to interact with the recombinant opioid receptor polypeptide involves measuring (i) [binding ability; (ii)] the ability of the recombinant opioid receptor polypeptide to bind the candidate substance; [(iii)] (ii) ability of the candidate substance to activate ion channels in a cell membrane; or [(iv)] (ii) modulation of ion channels in the cell membrane of part (ii).

76. (Amended) The process of claim 74 [75], wherein the nucleic acid sequence comprises at least 40 contiguous nucleotides of SEQ ID NO:7, including the guanine nucleotide at position 389 of SEQ ID NO:7.

77. (Amended) The process of claim 74 [75], wherein the nucleic acid sequence comprises at least 45 contiguous nucleotides of SEQ ID NO:7, including the guanine nucleotide at position 389 of SEQ ID NO:7.

78. (Amended) The process of claim 74 [75], wherein the nucleic acid sequence comprises at least 50 contiguous nucleotides of SEQ ID NO:7, including the guanine nucleotide at position 389 of SEQ ID NO:7.

79. (Amended) The process of claim 74 [75], wherein the nucleic acid sequence comprises at least 75 contiguous nucleotides of SEQ ID NO:7, including the guanine nucleotide at position 389 of SEQ ID NO:7.

80. (Amended) The process of claim 74 [75], wherein the nucleic acid sequence comprises at least 100 contiguous nucleotides of SEQ ID NO:7, including the guanine nucleotide at position 389 of SEQ ID NO:7.

81. (Amended) The process of claim 74 [75], wherein the nucleic acid sequence comprises the nucleotide sequence of SEQ ID NO:7, including the guanine nucleotide at position 389 of SEQ ID NO:7.

82. (Amended) The process of claim 74 [75], wherein recombinant opioid receptor polypeptide is chimeric.

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